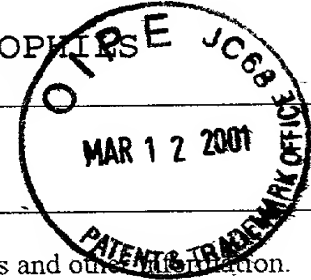


U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER <b>JULIAN 1</b>
<b>TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371</b>		U.S. APPLICATION NO. (If known, see 37 CFR 1.5) <b>09/787006</b>
INTERNATIONAL APPLICATION NO. <b>PCT/FR99/02145</b>	INTERNATIONAL FILING DATE <b>09 September 1999</b>	PRIORITY CLAIMED <b>10 September 1998</b>
TITLE OF INVENTION <b>METHOD FOR DETECTING OR QUANTIFYING BASOPHILS AND EOSINOPHILS</b>		
APPLICANT(S) FOR DO/EO/US <b>Felix MONTERO JULIAN et al.</b>		
<p>Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.</p> <ol style="list-style-type: none"> <li><input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li><input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li><input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).</li> <li><input checked="" type="checkbox"/> The US has been elected in a Demand by the expiration of 19 months from the priority date (PCT Article 31).</li> <li><input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))             <ol style="list-style-type: none"> <li><input type="checkbox"/> is attached hereto (required only if not transmitted by the International Bureau).</li> <li><input checked="" type="checkbox"/> has been communicated by the International Bureau</li> <li><input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</li> </ol> </li> <li><input checked="" type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).</li> <li><input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))             <ol style="list-style-type: none"> <li><input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</li> <li><input type="checkbox"/> have been communicated by the International Bureau.</li> <li><input type="checkbox"/> have not been made, however, the time limit for making such amendments has NOT expired.</li> <li><input checked="" type="checkbox"/> have not been made and will not be made.</li> </ol> </li> <li><input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</li> <li><input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</li> <li><input type="checkbox"/> An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</li> </ol> <p><b>Items 11. to 16. below concern document(s) or information included:</b></p> <ol style="list-style-type: none"> <li><input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</li> <li><input type="checkbox"/> An Assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</li> <li><input type="checkbox"/> A FIRST preliminary amendment.</li> <li><input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</li> <li><input type="checkbox"/> A substitute specification.</li> <li><input type="checkbox"/> A change of power of attorney and/or address letter.</li> <li><input checked="" type="checkbox"/> Other items or information.             <ul style="list-style-type: none"> <li><input checked="" type="checkbox"/> Courtesy copy of the first page of the International Publication (WO 00/16103).</li> <li><input checked="" type="checkbox"/> Courtesy copy of the International Preliminary Examination Report. There were no annexes</li> <li><input checked="" type="checkbox"/> Formal drawings, 9 sheets, Figures 1-7.</li> <li><input checked="" type="checkbox"/> Courtesy Copy of the International Search Report.</li> </ul> </li> </ol>		



U.S. APPLICATION NO (If known, see 37 CFR 1.5) <b>09/787006</b>		International Application No <b>PCT/FR99/02145</b>		Attorney's Docket No <b>JULIAN 1</b>	
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<p>17. [xx] The following fees are submitted.</p> <p><b>BASIC NATIONAL FEE (37 CFR 1.492 (a)(1) - (5):</b></p> <p>Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO.....\$1000.00</p> <p>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO.....\$860.00</p> <p>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....\$710.00</p> <p>International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4).....\$690.00</p> <p>International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4).....\$100.00</p> <p style="text-align: center;"><b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b></p> <p>Surcharge of \$130.00 for furnishing the oath or declaration later than [ ] 20 [X] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).</p> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 30%;">Claims as Originally Presented</th> <th style="width: 15%;">Number Filed</th> <th style="width: 15%;">Number Extra</th> <th style="width: 10%;">Rate</th> <th style="width: 10%;"></th> <th style="width: 10%;"></th> </tr> <tr> <td>Total Claims</td> <td>18 - 20</td> <td></td> <td>X \$18.00</td> <td>\$</td> <td></td> </tr> <tr> <td>Independent Claims</td> <td>2 - 3</td> <td></td> <td>X \$80.00</td> <td>\$</td> <td></td> </tr> <tr> <td colspan="4">Multiple Dependent Claims (if applicable)</td> <td>+\$270.00</td> <td>\$ 270.00</td> </tr> <tr> <td colspan="4" style="text-align: center;"><b>TOTAL OF ABOVE CALCULATIONS =</b></td> <td></td> <td>\$1,260.00</td> </tr> </table> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 30%;">Claims After Post Filing Prel. Amend</th> <th style="width: 15%;">Number Filed</th> <th style="width: 15%;">Number Extra</th> <th style="width: 10%;">Rate</th> <th style="width: 10%;"></th> <th style="width: 10%;"></th> </tr> <tr> <td>Total Claims</td> <td>- 20</td> <td></td> <td>X \$18.00</td> <td>\$</td> <td></td> </tr> <tr> <td>Independent Claims</td> <td>- 3</td> <td></td> <td>X \$78.00</td> <td>\$</td> <td></td> </tr> <tr> <td colspan="4" style="text-align: center;"><b>TOTAL OF ABOVE CALCULATIONS =</b></td> <td></td> <td>\$1,260.00</td> </tr> </table> <p>Reduction of 1/2 for filing by small entity, if applicable. Applicant claims small entity status. See 37 CFR 1.27.</p> <p style="text-align: center;"><b>SUBTOTAL =</b></p> <p>Processing fee of \$130.00 for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f))</p> <p style="text-align: center;"><b>TOTAL NATIONAL FEE =</b></p> <p>Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +</p> <p style="text-align: center;"><b>TOTAL FEES ENCLOSED =</b></p> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 60%;"></td> <td style="width: 20%; text-align: center;">Amount to be:</td> <td style="width: 20%; text-align: center;">\$</td> </tr> <tr> <td></td> <td style="text-align: center;">refunded</td> <td></td> </tr> <tr> <td></td> <td style="text-align: center;">charged</td> <td style="text-align: center;">\$</td> </tr> </table>				Claims as Originally Presented	Number Filed	Number Extra	Rate			Total Claims	18 - 20		X \$18.00	\$		Independent Claims	2 - 3		X \$80.00	\$		Multiple Dependent Claims (if applicable)				+\$270.00	\$ 270.00	<b>TOTAL OF ABOVE CALCULATIONS =</b>					\$1,260.00	Claims After Post Filing Prel. Amend	Number Filed	Number Extra	Rate			Total Claims	- 20		X \$18.00	\$		Independent Claims	- 3		X \$78.00	\$		<b>TOTAL OF ABOVE CALCULATIONS =</b>					\$1,260.00		Amount to be:	\$		refunded			charged	\$	<p style="text-align: center;"><b>CALCULATIONS PTO USE ONLY</b></p>	
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a. [ ] A check in the amount of \$\_\_\_\_\_ to cover the above fees is enclosed

b. [X] Credit Card Payment Form (PTO-2038), authorizing payment in the amount of \$ 1,260.00, is attached.

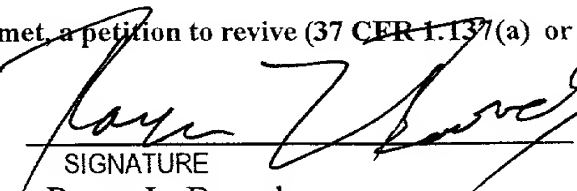
c. [ ] Please charge my Deposit Account No. **02-4035** in the amount of \$\_\_\_\_\_ to cover the above fees.  
A duplicate copy of this sheet is enclosed.

d. [XX] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **02-4035**. A duplicate copy of this sheet is enclosed.

**NOTE:** Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

**BROWDY AND NEIMARK, P.L.L.C.**  
**624 NINTH STREET, N.W., SUITE 300**  
**WASHINGTON, D.C. 20001**  
**TEL: (202) 628-5197**  
**FAX: (202) 737-3528**  
 Date of this submission: **March 12, 2001**

  
 SIGNATURE  
**Roger L. Browdy**  
 NAME  
**25,618**  
 REGISTRATION NUMBER

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A process for the detection or quantification of basophils and eosinophils

5 The present application relates to a novel process for the detection or quantification of basophils and eosinophils in healthy or sick persons, the processes for the preparation of the reagents required and the implementation thereof, particularly in the form of kits.

10 It is difficult to study eosinophils and basophils because of the very small number of these cells in the blood, that is, from 1% to 3% and less than 1% respectively. However, their number may increase considerably in certain pathological states such as parasitic infections, allergic and asthmatic conditions or certain leukaemias.

15 It has been shown that eosinophils play a very important part in chronic allergic asthma. Eosinophils infiltrate the respiratory passages where they may be activated and may degranulate. The release of the granular contents, enzymes and basic proteins causes damage to the bronchial epithelium. Some reports also mention eosinophil dysfunction.

The bridging of specific IgEs present on basophils by various allergens such as pneumoallergens, venoms, food proteins or medicinal products may lead to the release of the mediators contained in the granules or neoformed and capable of inducing anaphylactic shock.

20 In order to understand the condition of an individual and to assess his susceptibility to exogenous agents, it is important therefore to count the eosinophils and basophils and to measure the proportion of those which are capable of activation by a particular agent.

25 The methods of counting basophils and eosinophils are based on staining the granules (manual counting) with, for example, ortho-toluidine blue for basophils and with eosine for eosinophils, their size and their particle size distribution, or based on differential immunophenotyping of their cell surface. These methods are unwieldy, time-consuming and not very specific. They have a high level of uncertainty and considerable statistical errors because of the small number of these cell types.

Moreover, most of these methods are unsuitable for analysing the degree of activation of these cells.

For all these reasons, it would be desirable to have products and processes by means of which eosinophils and basophils can be detected and quantified more specifically, whether or not they have been activated.

Various processes using a flow cytometer have been described for the detection of eosinophils and basophils (see, for example, Nakagawa et al., (1981) Allergy, 36; pp 39-47; Gane et al., (1995) Cytometry 19; pp 361-365; Hübl et al., (1996) J. Clin. Lab. Analysis 10, 177-183).

One of the main problems faced when studying basophils and eosinophils by flow cytometry is the distinct separation between the positive cells and the negative cells. Some authors use a remarkable characteristic of eosinophils, their autofluorescence, to identify them more precisely. However, certain clinical samples exhibit very high autofluorescence of monocytes, neutrophils and lymphocytes. The great variability in the particle size distribution of eosinophils should also be emphasised here, this being reflected in the fact that the zone of the scatter diagram (distribution as a function of size and particle size distribution) corresponding to these cells is spread out. On the scatter diagram, the basophils are situated on the border between the lymphocytes and monocytes.

Leucocytes express proteins on their surface characterised in terms of CDs (Cluster of Differentiation) by groups of antibodies which recognise them. The expression of several CDs on the same cell may enable the cell to be characterised. The same CD may be expressed on different cell types. For this reason, most of the flow cytometry processes use mixtures of antibodies that recognise several of these CDs as markers of basophils and eosinophils.

For example, the use of two antibody mixtures, anti-CD2, anti-CD14, anti-CD16 and anti-CD19 on the one hand, and anti-CD32, anti-CD25, anti-IgG1 and anti-IgG4 on the other hand makes it possible to detect basophils with a flow cytofluorimeter. The anti-CD2 antibodies detect T cells, the anti-CD19 antibodies make it possible to detect B cells, and the anti-CD14 antibodies detect mainly monocytes but also granulocytes.

CD16, an IgG type III receptor, is expressed on neutrophils, certain T (NK) cells and on monocytes. CD32, an IgG type II receptor, is expressed on monocytes, neutrophils and B lymphocytes. CD25 is a marker of T and B cell activation, and a marker of macrophage activation. Several of these markers are shared by different types of leucocytes. Basophils and eosinophils are minority populations of leucocytes. For this reason, most of the data on basophils and eosinophils have been obtained from purified cells. For example, it was possible to study the basophils of individuals with chronic myeloid leukaemia after several purification steps using monoclonal antibodies followed by lysis of the red corpuscles by complement. However, this process is impracticable for routine use because it is long and requires a large volume of blood.

Other processes for the detection of basophils use an anti-IgE antibody or an anti-receptor antibody with a high affinity for IgE. These processes do not, however, permit a clear identification of basophils because small amounts of high-affinity IgE receptor have been revealed on monocytes, and eosinophils and B cells may carry IgE on their surface. Moreover, bridging of IgE or of their receptors on the surface of basophils by the antibodies used as a probe may lead to cell activation altering the properties of the membrane. It has been shown that the activation of basophils by an allergen brings about a reduction in the binding of an anti-IgE antibody.

It would be desirable, therefore, to be able to count eosinophils and basophils easily and without risk of error, even amongst the other cell populations of human blood, and to measure the proportion of those capable of being activated by a particular agent.

In response to the problem of diagnosing and treating diseases such as chronic bronchial asthma or atopic dermatitis, EP-A-0 811 691 proposes antibodies of various types: monoclonal, humanised etc., directed against the  $\alpha$  chain of the interleukin-5 receptor which blocks the activity of this cytokine. It also proposes a process for the detection of eosinophils. But the use of antibodies neutralising the biological activity of IL-5 gives disappointing results in the flow-cytometry analysis. The specific signal

obtained on eosinophils, even if they have been purified, cannot be distinguished from the signal obtained with a control antibody (background).

When characterising a group of monoclonal antibodies directed against the interleukin-5 receptor (IL-5R), the Applicant discovered that, surprisingly, basophils and eosinophils amongst other cell populations of human or animal blood could be detected in isolation and together by using certain antibodies specific for the alpha chain of the interleukin-5 receptor and that these same cells could also be counted, if desired. The Applicant has shown, by bio-assays on TF1 cells which are dependent on IL-5 for their survival, that these selected antibodies did not inhibit the growth of these cells.

Moreover, the Applicant has also shown that the receptor bound to these antibodies and immobilised on a solid phase is still capable of fixing marked IL-5.

For this reason, the present application provides a process for the detection or quantification of eosinophils and basophils, characterised in that it comprises bringing a sample optionally containing said eosinophils or basophils into contact with an IL-5 anti-receptor (alpha chain) monoclonal antibody which does not interfere with the fixing of IL-5 to its receptor and which does not inhibit the biological activity of IL5 in order to detect and, if desired, quantify the eosinophils and basophils.

The invention uses, therefore, an IL-5 anti-receptor (alpha chain) monoclonal antibody and the expression of the alpha chain of the IL-5 receptor as a specific marker of eosinophils and basophils. It was observed that this marking was specific for these blood cells and that none of the B or T cells, monocytes or neutrophils was marked.

These antibodies may therefore be used for counting basophils and eosinophils accurately and precisely under all circumstances.

The sample optionally containing said eosinophils or basophils may be, for example, a blood sample originating in particular from a sick person, preferably suffering from an allergy or parasitic disorder.

According to the process described above, the IL-5 anti-receptor monoclonal antibody is an antibody which does not interfere with the fixing of IL-5 to its receptor and which does not inhibit the biological activity of IL-5.

The absence of inhibition of the biological activity of IL-5 may be shown by bio-assay on TF1 cells which are dependent on IL-5 for their survival, the antibodies according to the invention not inhibiting the growth of these TF1 cells.

5 Under preferred conditions of carrying out the process described above, the IL-5 anti-receptor monoclonal antibody is an antibody which does not interfere with IgE. The expression "does not interfere with IgE" means an antibody which does not prevent the binding of an allergen or of another anti-IgE antibody to these surface IgE.

10 Under further preferred conditions of carrying out the process described above, the IL-5 anti-receptor monoclonal antibody is an antibody which does not interfere with cell activation of eosinophils or basophils. The expression "does not interfere with cell activation of eosinophils or basophils" means an antibody which, by virtue of its binding to the surface of the cell, does not induce or inhibit the appearance of a surface activation marker of basophils or eosinophils.

15 Under yet further preferred conditions of carrying out the process described above, the detection and, if desired, the quantification of eosinophils and basophils uses a flow cytometer or optical scanning cytometer.

Human basophils express on their surface markers such as CD11, CD13, CD18, CD26, CD31, CD32, CD33, CD40, CD43, CD44, CD45, CD49d, CD54, and eosinophils express on their surface CD15, CD32, CD44, CD49d, CD52, CD65, CD66, 20 CD67. On the other hand, neither basophils nor eosinophils express CD3, CD14, CD16, CD19. These lists of markers are not exhaustive.

For this reason, the present application also provides an above process characterised in that, moreover, the sample is brought into contact with one or preferably more than one other monoclonal antibody directed against other markers 25 making it possible to exclude cell types other than eosinophils or basophils. Thus, it is possible to distinguish lymphocytes, monocytes and neutrophils from eosinophils and basophils.

Exclusion markers are therefore used to improve still further the accuracy of counting by excluding erratic events. The present application provides, in particular, an

above process characterised in that the other monoclonal antibodies are directed against the markers CD3, CD16 and CD19 which make it possible to exclude erratic events.

The invention permits the detection and specific quantification of activated or non activated basophils and eosinophils. It has been shown recently that CD63, a lysosomal protein of the tetraspan family and initially described as a platelet activation marker, was also present in the granules of basophils and neutrophils. The expression of CD63 on the cell surface is calcium-dependent. When basophils are activated, the CD63 expressed on the surface may be recognised by a specific antibody. The intensity of marking is a function of the number of cells activated.

For this reason, the present application also provides an above process characterised in that the detection or quantification of activated basophils is carried out by also bringing the sample into contact with one or more other monoclonal antibodies directed against basophil activation markers and particularly against the CD63 antigen.

Similarly, the present invention also provides an above process characterised in that the detection or quantification of activated eosinophils is carried out by also bringing the sample into contact with one or more monoclonal antibodies directed against eosinophil activation markers and more particularly against the CD69 antigen.

Moreover, it is possible to detect and count basophils and eosinophils separately if an additional means of discrimination is available such as

- the diffusion of light in flow cytometers where eosinophils are distinguished from basophils by a much higher side scatter value,
- the peroxidase activity characteristic of eosinophils which is revealed by using precipitating substrates such as DAB (diaminobenzidine) or fluorescent substrates such as dichlorofluorescein diacetate, a non-fluorescent substrate converted to highly fluorescent 2',7'-dichlorofluorescein.

The present application also provides an anti-IL-5R antibody characterised by:

- the absence of interference with the fixing of IL-5 to its receptor,
- the absence of interference with IgE,
- the absence of interference with cell activation of eosinophils or basophils,
- the absence of inhibition of the biological activity of IL-5.



5 An advantageous embodiment of the invention consists in the use of a mixture of antibodies directed, on the one hand, against IL-5R, with or without "specific" markers for T and B lymphocytes, NK cells, monocytes and neutrophils and, if activated eosinophils or basophils are of interest, on the other hand, a marker of basophil and eosinophil activation. The cell activation marker may be a specific antibody for a protein appearing on the surface of the cell membrane after activation, or the detection of oxidative enzyme activity. The latter makes it possible to detect and, optionally, to quantify cell activation.

10 For this reason, the present application also provides kits targeted for different applications, said kits having, as their basis, an anti-IL-5R monoclonal antibody, preferably of mouse, rat or rabbit, or genetically modified, and primarily

- 15 a kit for the detection or quantification of eosinophils or basophils containing
  - at least one anti-IL-5R monoclonal antibody as defined above conjugated to a first fluorochrome,
  - a mixture of antibody markers for lymphocytes, monocytes and neutrophils, conjugated to a second fluorochrome,
- and a kit for the detection and quantification of activated eosinophils and basophils containing
  - 20 - at least one anti-IL-5R monoclonal antibody as defined above conjugated to a first fluorochrome,
  - a mixture of antibody markers for lymphocytes, monocytes and neutrophils, conjugated to a second fluorochrome and
  - antibodies directed against activation markers and conjugated to a third fluorochrome.

25 The present application also provides an above kit, characterised in that it also includes a specific substrate for the oxidative activity of eosinophils.

The present application also provides a kit for the detection or quantification of the oxidative activity of eosinophils or basophils containing

- 30 - at least one anti-IL-5R monoclonal antibody as defined above conjugated to a first fluorochrome,

- a mixture of antibody markers for lymphocytes, monocytes and neutrophils conjugated to a second fluorochrome,

a marker substrate for the oxidative activity of eosinophils or basophils.

The preferred conditions for carrying out the processes described above also apply to the other subject matter of the invention mentioned above.

Finally, the present application provides an above process, antibody or kit, characterised in that the IL-5 anti-receptor monoclonal antibody is an antibody of the IgG1 type, the corresponding hybridoma of which was lodged with the Collection Nationale de Culture de Micro-organismes (CNCM) under no. 1-2068.

The invention may be applied in the test, by clinical and pharmaceutical laboratories, of the response of basophils or eosinophils to different types of degranulating agents (allergens, chemicals, parasites etc) in order to make a diagnosis or to monitor desensitisation, or to reveal the capacity of new molecules to modify the degranulation of basophils or eosinophils and for studying allergic, parasitic and leukaemic pathologies.

The examples below illustrate the present application.

Figure 1 is an illustration of a dot plot obtained with the anti-IL-5R H17N (B) monoclonal antibody and an isotype control (A) conjugated to phycoerythrin. The abscissas represent the intensity of fluorescence expressed on a logarithmic scale, and the ordinates represent side scatter.

The polygons labelled R4 and R5 represent basophils and eosinophils respectively.

Figures 2A and 2B illustrate a double marking obtained with the anti-IL-5R antibody conjugated to phycoerythrin for basophils and eosinophils respectively. The abscissas represent the fluorescence emitted by fluorescein (FITC) and the ordinates the fluorescence emitted by phycoerythrin (PE).

The left-hand dot plot represents the double marking between the anti-IL-5R-PE and an antibody specific for another marker conjugated to FITC. The right-hand dot plot represents the marking obtained with an isotype control of the anti-IL-5R-PE.

5 The markers used are, from top to bottom, anti-CD16 (this marker is present on neutrophils, NK cells, Kupffer cells and a sub-population of macrophages), anti-CD49d (alpha 4 chain of integrins expressed on basophils and eosinophils but also on lymphocytes, monocytes and thymocytes).

10 Figure 3 illustrates a double marking obtained with an anti-IL-5R antibody conjugated to phycoerythrin in combination with a mixture of CD3, CD19 and CD16 antibodies, all conjugated to phycoerythrin-cyanine 5 (PECy5). The FL-1 channel corresponds to the fluorescence emitted by fluorescein, channel FL2 corresponds to the fluorescence emitted by phycoerythrin and channel FL3 corresponds to the fluorescence emitted by phycoerythrin cyanine 5.

15 Diagram A corresponds to the scatter according to size and particle size distribution. Region R1 which corresponds to lymphocytes, region R2 which corresponds to monocytes and region R3 which corresponds to granulocytes can be distinguished.

20 Diagram B corresponds to the fluorescence of the anti-IL-5R-PE antibody as a function of side scatter, a function of cell size. R4 corresponds to basophils and R5 to eosinophils.

Diagram C represents the fluorescence of a mixture of anti-CD3, anti-CD16, anti-CD19 antibodies as a function of side scatter. R6 corresponds to unmarked eosinophils.

25 Diagram D represents the fluorescence of the mixture of anti-CD3, anti-CD16 and anti-CD19 antibodies and the fluorescence of the anti-IL-5R-PE antibody. The eosinophils project into the regions R5 and R6. Only the events present in R5 and R6 are taken into account in diagram D.

30 Figure 4 illustrates the correlation between the percentages given by a haematology analyser and the process of the invention. The abscissas represent the percentage of eosinophils (A) and the percentage of basophils (B) obtained in flow

cytometry and the ordinates represent the percentage of eosinophils (A) and the percentage of basophils (B) given by the haematology analyser.

The top graph corresponds to the data obtained for the eosinophils and the bottom graph to the data obtained for basophils. The correlation coefficients are given inside each graph.

Figure 5 is a typical illustration of a result obtained after activation of the basophils with an anti-IgE antibody.

On this figure 5, dot plot 1 represents the size and particle size distribution of the blood cells, diagram 2 represents the marking obtained with the anti-IL-5R-PE antibody (diagram similar to that shown in Figure 1), diagram 3 represents the isolation of the basophils and the removal from the population of other possible contaminants such as lymphocytes and monocytes. Diagram 4 represents basophils.

Figure 5B represents 4 dot plots of activated basophils. Each dot plot corresponds to the stimulation of whole blood by decreasing concentrations of anti-IgE. The cloud of dots of each dot plot corresponds to the population, isolated according to the dot plot 5A-4. The abscissas represent the fluorescence emitted by fluorescein conjugated to the anti-CD63 antibody, and the ordinates represent the fluorescence emitted by phycoerythrin conjugated to the anti-IL-5R antibody.

Figure 6 illustrates the comparison between the percentages of the CD63+IL-5R+ cells and the percentage of histamine released.

Each diagram represents a donor. The black symbols represent the % of CD63+IL-5R+ cells and the white symbols the percentage of histamine released. The abscissas represent the concentration of anti-IgE used by stimulation of the basophils expressed in  $\mu\text{g/ml}$  and the ordinates represent the percentage of basophils expressing CD63 or the percentage of histamine released.

Figure 7 illustrates the activation of eosinophils by an ester of phorbol (PMA: phorbol myristate acetate). The abscissas represent the concentration of PMA added and the ordinates represent the percentage of eosinophils expressing the CD69 or CD63 molecule. Each diagram represents a different donor.

**EXAMPLE 1 . Obtaining anti-IL-5R monoclonal antibodies****1. Reagents**

Common reagents (salts, buffers etc.) were purchased from Merck, Darmstadt, Germany. The cell culture reagents originate from Biowittaker Virviers, Belgium and from Sigma, Saint-Louis, USA. The anti-CD3, anti-CD16, anti-CD19, anti-CD49d, anti-CD63, anti-CD69 monoclonal antibodies conjugated to fluorescein N-isothiocyanate (FITC) or to phycoerythrin cyanine 5 (PECy5) and the anti-IgE monoclonal antibody clone E124.2.8 are commercial products obtained from Immunotech, Marseilles, France.

**2. Fusion**

The anti-IL-5R antibody, clone H17N, was obtained after immunisation of mice with a soluble recombinant form of IL-5R, a protein produced from a gene fusion IL-5R - IL-2 and expressed in the CHO cells, then fusion of the blastocytes with the cells of myeloma X63 using the conventional technique described by Köhler and Milstein (1975, Nature 256, 495). The production of antibodies was screened by ELISA using plates covered either with hybrid protein IL-5R - IL-2 or with IL-2. The positive clones on the plates covered with IL-5R - IL-2 and negative clones on plates covered with IL-2 were cloned by limiting dilution.. The antibodies were selected on TF-1 cells by virtue of their ability to recognise IL-5R in the presence and in the absence of IL-5, the criterion according to the invention.

Of the monoclonal antibodies selected, the anti-IL-5R monoclonal antibody known as H17N has high affinity for the antigen but does not inhibit the biological activity of IL-5. Consequently, despite the binding of IL-5 to its receptor, no inhibition is observed. The corresponding hybridoma was lodged with the Collection Nationale de Culture de Micro-organismes (CNCM) in Paris on 3 September 1998 under no. 1-2068. The anti-IL-5R monoclonal antibody known as H17N is of the IgG1 type.

The anti-IL-5R antibody H17N was conjugated to phycoerythrin according to the protocol described in "Bioconjugate techniques" by G. Hermanson, Academic Press, 1996.

## 5           **EXAMPLE 2. Analysis by cytometry on whole blood**

### 1. Blood samples

10           The blood samples used for the correlation analysis were obtained from Hôpital La Conception (Marseilles, France). The blood formulation was carried out on tubes containing EDTA as anticoagulant with an STKS-Coulter apparatus, Miami, USA. The blood used for basophil activation was taken using heparin tubes. These samples were taken from persons belonging to the laboratory.

### 2. Protocol

15           The expression of IL-5R in different cell sub-populations, from whole blood, was analysed by dual immunolabelling using, on the one hand, anti-IL-5R H17N antibody conjugated to phycoerythrin prepared in stage 3 of example 1 and, on the other hand, markers specific for the cell populations studied.

20           The protocol used was as follows: 100 µl of whole blood were incubated with 20 µl of anti-IL-5R antibody H17N-PE and 20 µl of a second specific antibody for another marker. The sample was incubated for 15 min at ambient temperature.

25           Then 1 ml of lysis reagent was added and the reaction mixture mixed vigorously immediately. When the sample became translucent (<1 min), 250 µl of fixing reagent were added and the sample was again mixed carefully. Three ml of isotonic phosphate buffer (PBS 1X) were then added, then the sample was centrifuged for 3 minutes at 1200 rpm. The supernatant was removed by suction and 500 µl of PBS-0.5% formaldehyde were added. The samples were stored at 4 °C in the dark before the cytometer analysis.

Non-specific binding was monitored using an irrelevant antibody of the same isotype reference IM 0670 (Immunotech, Marseilles). This makes it possible to carry out appropriate adjustments to the device. The cytometric analysis of the cells labelled by different fluorochromes was carried out on an FACScalibur apparatus (Becton Dickinson, Mountain View, USA). 100,000 events were analysed for each sample.

### 3. Identification of basophils and eosinophils by flow cytometry

The anti-IL-5R H17N antibody was conjugated to phycoerythrin (PE) in order to characterise more easily the cells which express the IL-5 receptor. The immunolabelling of whole blood with H17N-PE antibody and a control antibody (irrelevant antibody of the same isotype reference IM 0670) is shown in Figure 1 (A and B).

In the presence of the control antibody, an absence of marking was observed (Figure 1A). On the other hand, in the presence of the anti-IL-5R-PE antibody, a time lag of the fluorescence of certain cells was observed (Figure 1B). The two clouds (windows R5 and R4) of the cells marked by the anti-IL-5R antibody correspond to the eosinophils and basophils. This observation was confirmed thanks to several immunolabelling operations using antibodies specific for different CDs (Clusters of Differentiation). Thus, it was observed that the cells which were positive for IL-5R are negative for the expression of CD3, CD19 and CD16, these CDs being specific for lymphocytes, and negative for the expression of CD14, which is specific for monocytes. However, the cells are positive for CD49d, a protein expressed by eosinophils and basophils, but also by lymphocytes, monocytes and thymocytes.

This shows that the cells marked by the anti-IL-5R antibody correspond well to eosinophils and basophils.

### EXAMPLE 3: Quantification of eosinophils and basophils

The difference between the percentage of eosinophils and basophils given by the STKS haematology analyser (Coulter, Miami), used in the Hôpital de la Conception in Marseilles, was compared with the percentage of cells which were positive for the

expression of IL-5R, detected by the anti-IL-5R H17N antibody according to the invention.

Basophils and eosinophils are the only types of cells marked by the anti-IL-5R antibody according to the invention, but the cloud of basophils is very close to the cloud of cells corresponding to lymphocytes.

A mixture of anti-CD3, anti-CD16 and anti-CD19 antibodies conjugated to phycoerythrin cyanine 5 was added to the anti-IL-5R antibody conjugated to PE, which made it possible to exclude erratic events. This combination made it possible to obtain more accurate values. By using this method, the percentages of eosinophils and basophils obtained by the two methods for 50 different samples were compared. The results are shown in Figure 4.

Figure 4A shows the correlation of the percentages of eosinophils obtained with both techniques. This is  $[\% \text{ haematology analyser}] = 0.94 [\% \text{ cytometer}] + 0$  for  $n = 50$ . Figure 4B shows the correlation of percentages of basophils obtained with both techniques. This is  $[\% \text{ haematology analyser}] = 0.86 [\% \text{ cytometer}] + 0.076$  for  $n = 50$ . A very good correlation was observed between the two methods,  $r = 0.98$  and  $r = 0.83$ , showing the great reliability of the process according to the invention.

#### EXAMPLE 4: Activation of basophils

The release of histamine by the activated basophils was carried out on 300  $\mu\text{l}$  aliquots of blood placed in a tube and incubated in the presence of different concentrations of anti-IgE in order to establish a dose-response curve.

The samples were mixed gently and incubated at 37 °C for 15 minutes. Cell activation was stopped by adding 300  $\mu\text{l}$  of cold phosphate buffer containing 1 mM EDTA. The tubes were centrifuged at +4 °C for 3 minutes at 1200 rpm. The supernatant was recovered in order to quantify the histamine. The cell residue was resuspended in 300  $\mu\text{l}$  of phosphate buffer, 1 mM EDTA and 100  $\mu\text{l}$  of this cell suspension were analysed after immunolabelling.



Quantification of the histamine released was carried out using a commercial radioimmunoassay (reference 1659, Immunotech, Marseilles, France), following the manufacturer's instructions. Total histamine was determined after cell lysis by dilution of 50 µl of blood in 950 µl of distilled water followed by two freeze-defrost cycles. The histamine released after cell activation is expressed as a percentage of total histamine.

The analysis by flow cytometry was carried out after triple marking on 100 µl of cell residue taken up by PBS with anti-IL5R H17N-PE antibody, anti-CD63-FITC antibody and anti-CD3, anti-CD16, anti-CD19 antibodies, all three conjugated to PECy5. The procedure was identical to that described above. In the same way, the analysis of 100,000 cells was acquired. The percentage of doubly marked cells CD63+ and IL5R+ was determined after cell activation.

A representative experiment is shown in Figure 5. An increase in doubly marked cells (CD63 and IL-5R) as a function of the anti-IgE concentration was observed. This increase correlates perfectly with the profile of histamine released, as shown for four different donors in Figure 6.

#### EXAMPLE 5. Activation of eosinophils

The expression of the activation marker CD69 was carried out on 300 µl aliquots of blood placed in a tube and incubated in the presence of different concentrations of PMA (Phorbol Myristate Acetate) in order to establish a dose-response curve. The samples were mixed gently and incubated at 37 °C for 6 hours. Cell activation was stopped by centrifuging at +4 °C for 3 minutes at 1200 rpm. The supernatant was recovered in order to quantify the histamine. The cell residue was resuspended in 300 µl of PBS, 1 mM EDTA and 100 µl of this cell suspension were analysed after immunolabelling.

Quantification of the histamine released was carried out using a commercial radioimmunoassay (reference 1659, Immunotech, Marseilles, France), following the manufacturer's instructions. Total histamine was determined after cell lysis by dilution

of 50  $\mu$ l of blood in 950  $\mu$ l of distilled water followed by two freeze-defrost cycles. The histamine released after cell activation is expressed as a percentage of total histamine.

The analysis by flow cytometry was carried out after triple marking on 100  $\mu$ l of cell residue taken up by PBS with anti-IL-5R H17N-PE antibody, anti-CD69-FITC antibody or the anti-CD63-FITC antibody and the anti-CD3, anti-CD16, anti-CD19 antibodies, all three conjugated to PECy5. The procedure was identical to that described above. In the same way, the analysis of 100,000 cells was acquired. The percentage of doubly marked cells CD69+ and IL-5R+ on the one hand, CD63+ and IL5R+ on the other hand, was determined after cell activation.

A representative experiment is shown in Figure 7. An increase in doubly marked cells (CD69+ and IL-5R+) as a function of the PMA concentration is observed. Interestingly, the marking with the anti-CD63 antibody on the eosinophils did not give any fluorescence showing that CD63 is specific for basophils.

**EXAMPLE 6. Kit for the detection or quantification of eosinophils or basophils**

A kit corresponding to the following composition was prepared:

- a vial containing an anti-IL-5R H17N antibody conjugated to phycoerythrin,
- a vial containing a mixture of antibodies:
  - anti-CD3 conjugated to PECy5
  - anti-CD16 conjugated to PECy5
  - anti-CD19 conjugated to PECy5
- a vial containing an "isotype control" antibody conjugated to phycoerythrin
- a vial containing an "isotype control" antibody conjugated to PECy5

**EXAMPLE 7. Kit for the detection or quantification of activated eosinophils or basophils.**

A kit corresponding to the following composition was prepared:

- a vial containing an anti-IL-5R H17N antibody conjugated to phycoerythrin,

- a vial containing a mixture of antibodies:

anti-CD3 conjugated to PECy5

anti-CD16 conjugated to PECy5

anti-CD19 conjugated to PECy5

5

- a vial containing an anti-CD63 antibody conjugated to FITC,

- a vial containing an "isotype control" antibody conjugated to phycoerythrin

- a vial containing an "isotype control" antibody conjugated to PECy5

- a vial containing an "isotype control" antibody conjugated to FITC.

10

**EXAMPLE 8. Kit for the detection or quantification of eosinophils or basophils**

A kit corresponding to the following composition was prepared:

- a vial containing an anti-IL-5R H17N antibody conjugated to phycoerythrin,

- a vial containing a mixture of antibodies:

15

anti-CD3 conjugated to PECy5

anti-CD16 conjugated to PECy5

anti-CD19 conjugated to PECy5

- a vial containing an "isotype control" antibody conjugated to PECy5

- a vial containing dichlorofluorescein diacetate.

CLAIMS

1. A process for the detection or quantification of eosinophils and basophils, characterised in that it comprises bringing a sample optionally containing said eosinophils or basophils into contact with an IL-5 anti-receptor (alpha chain) monoclonal antibody which does not interfere with the fixing of IL-5 to its receptor and which does not inhibit the biological activity of IL-5 in order to detect and, if desired, to quantify the eosinophils and basophils.

2. A process according to claim 1, characterised in that the IL-5 anti-receptor monoclonal antibody is an antibody which does not interfere with IgE.

3. A process according to claim 1 or 2, characterised in that the IL-5 anti-receptor monoclonal antibody is an antibody which does not interfere with the cell activation of eosinophils or basophils.

4. A process according to one of claims 1 to 3, characterised in that the detection and, if desired, the quantification of eosinophils or basophils uses a flow cytometer or optical scanning cytometer.

5. A process according to one of claims 1 to 4, characterised in that, in addition, the sample is brought into contact with other monoclonal antibodies directed against other markers of the eosinophil or basophil cell types.

6. A process according to claim 5, characterised in that the other monoclonal antibodies are directed against the markers CD3, CD16 and CD19.

7. A process according to one of claims 1 to 6, characterised in that the detection or quantification of activated basophils is carried out by, in addition, bringing the sample into contact with one or more other monoclonal antibodies directed against basophil activation markers.

8. A process according to claim 7, characterised in that the activation marker is the CD63 antigen.

9. A process according to one of claims 1 to 6, characterised in that the detection or quantification of activated eosinophils is carried out by, in addition, bringing the

sample into contact with one or more other monoclonal antibodies directed against eosinophil activation markers.

10. A process for the detection and quantification of activated eosinophils according to claim 9, characterised in that the activation marker is the CD69 antigen.

5 11. An anti-IL-5R antibody characterised by:

- the absence of interference with the fixing of IL-5 to its receptor,
- the absence of interference with IgE,
- the absence of interference with cell activation of eosinophils or basophils,
- the absence of inhibition of the biological activity of IL-5.

10 12. A kit for the detection or quantification of eosinophils and basophils containing

- an anti-IL-5R monoclonal antibody according to claim 11 conjugated to a first fluorochrome,
- a mixture of antibody markers for lymphocytes, monocytes and neutrophils, conjugated to a second fluorochrome.

15

13. A kit for the detection and quantification of activated eosinophils and basophils containing

- an anti-IL-5R monoclonal antibody according to claim 11 conjugated to a first fluorochrome,

20

- a mixture of antibody markers for lymphocytes, monocytes and neutrophils, conjugated to a second fluorochrome and

- antibodies directed against activation markers and conjugated to a third fluorochrome.

25

14. A kit for the detection or quantification of the oxidative activity of eosinophils or basophils containing

- an anti-IL-5R monoclonal antibody according to claim 11 conjugated to a first fluorochrome,
- a mixture of antibody markers for lymphocytes, monocytes and neutrophils, conjugated to a second fluorochrome,

30

- a marker substrate for the oxidative activity of eosinophils or basophils.

15. A kit according to one of claims 12 to 14 applied to the study of allergic, parasitic or leukaemic pathologies.

16. A process, antibody or kit according to one of claims 1 to 15, characterised in that the IL-5 anti-receptor monoclonal antibody is an antibody of the IgG1 type, the  
5 corresponding hybridoma of which was lodged with the Collection Nationale de Culture de Micro-organismes (CNCM) under no. 1-2068.

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# ABSTRACT

The invention concerns a method for detecting or quantifying eosinophils and basophils, which consists in contacting a sample possibly containing said eosinophils or basophils with an IL-5 anti-receptor (alpha-chain) monoclonal antibody which does not interfere with IL-5 fixation to its receptor and does not inhibit the IL-5 biological activity to detect and, if required to quantify eosinophils and basophils. The invention also concerns a kit for detecting or quantifying eosinophils or basophils and an anti-IL5R antibody.

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ISOTYPIC CONTROL IgG1 PE

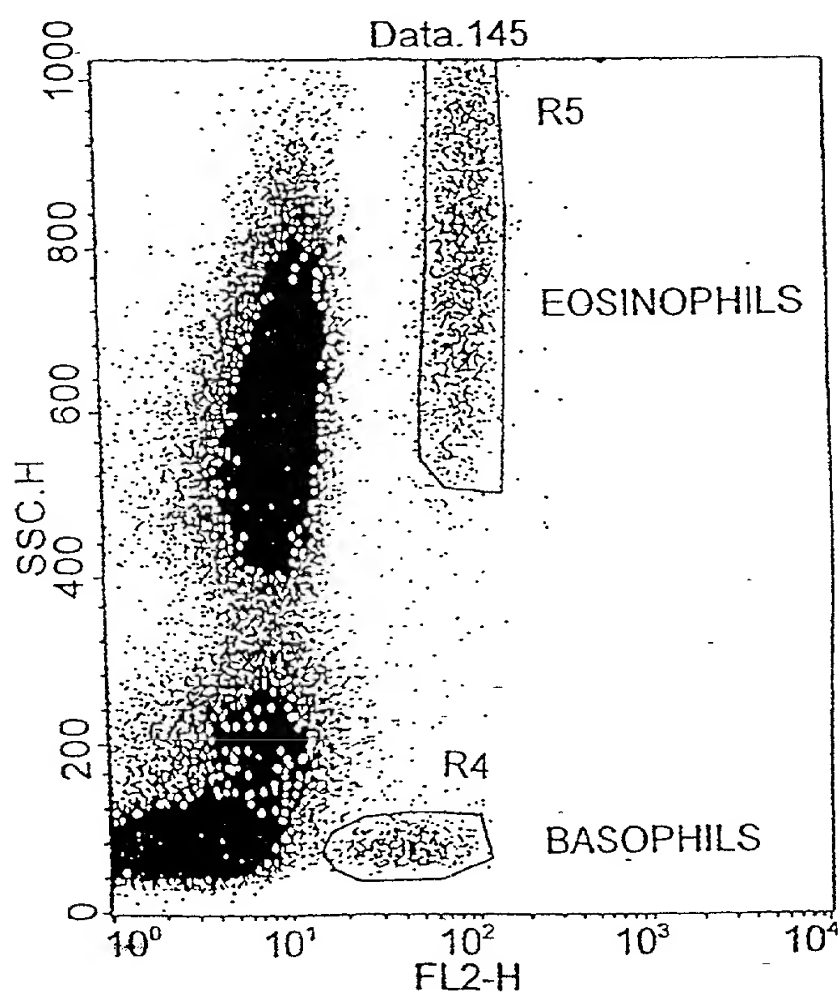
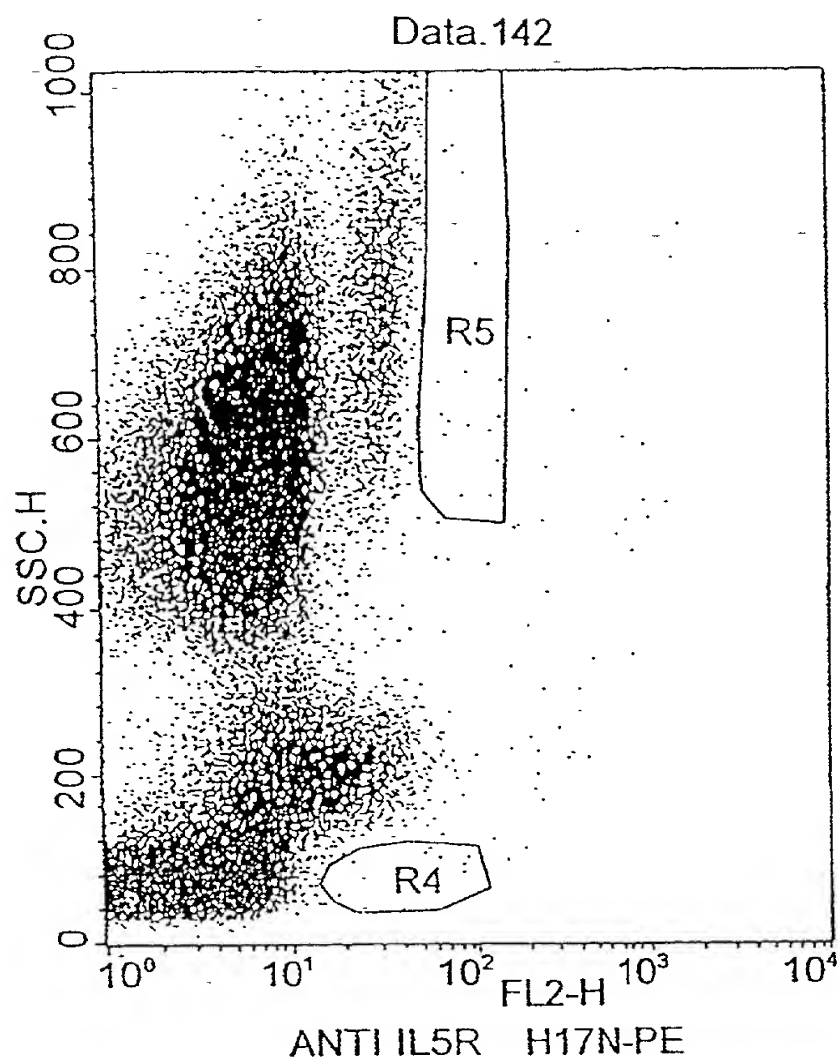


Fig. 1



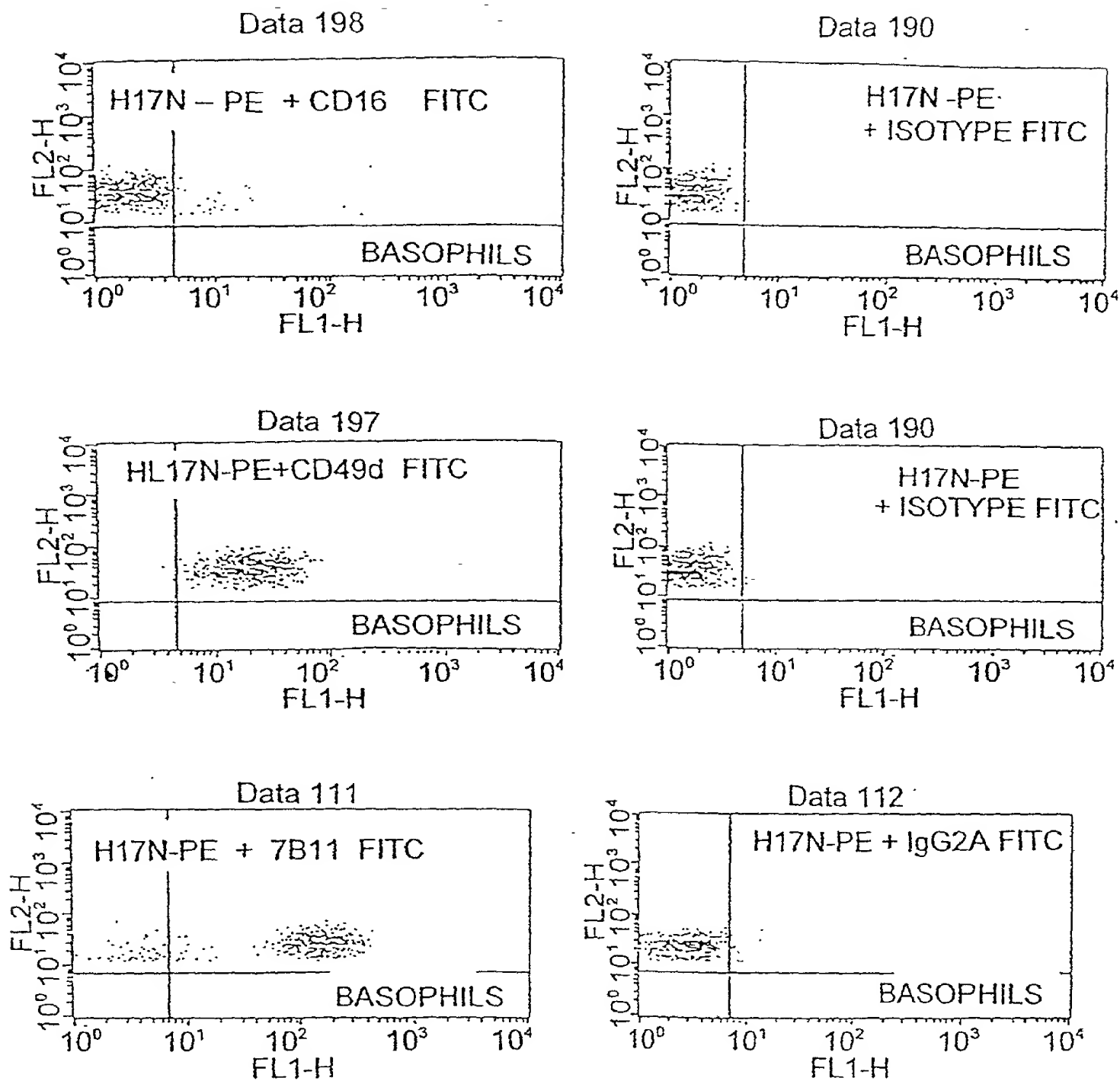


Fig. 2A

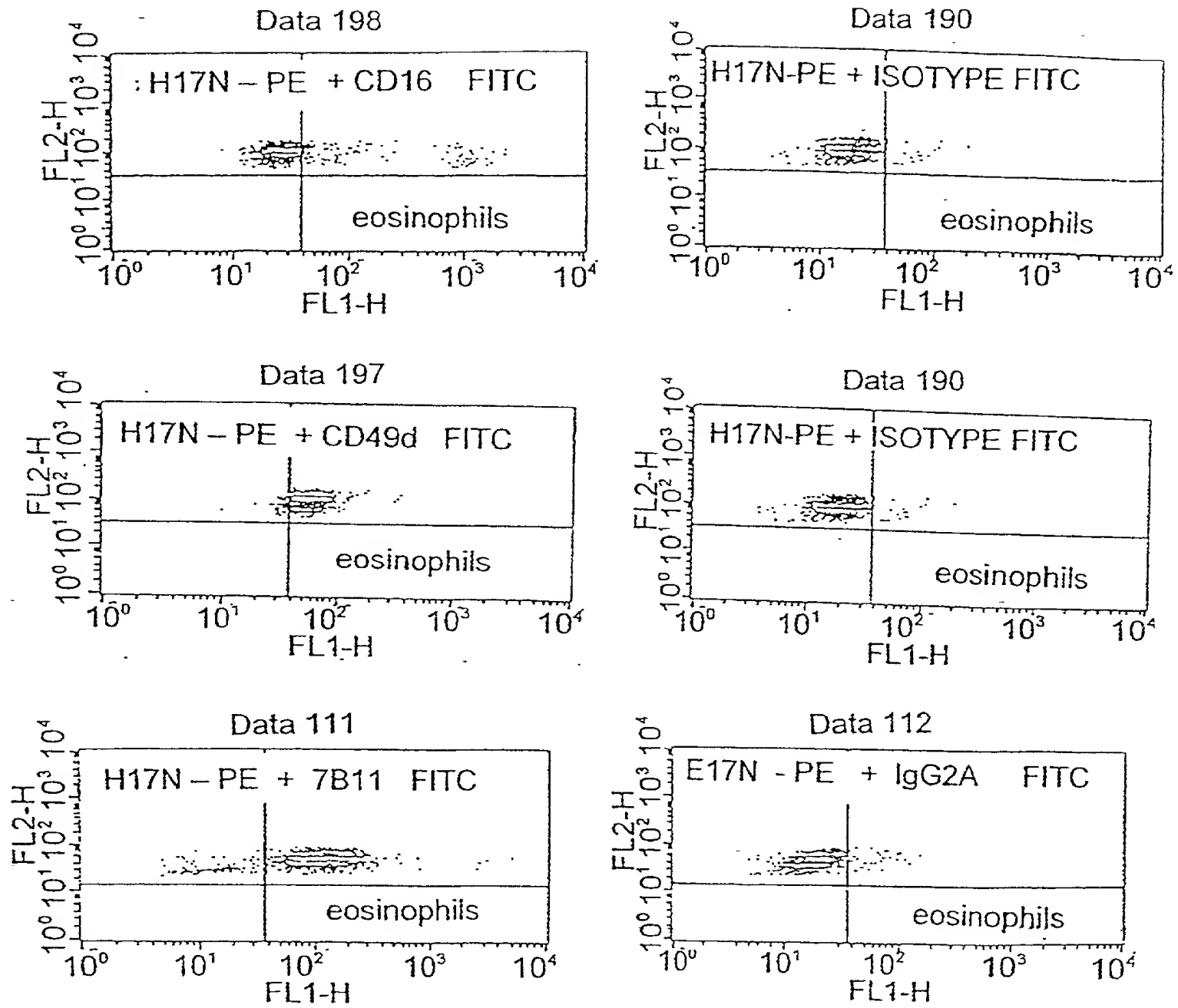


Fig. 2 B

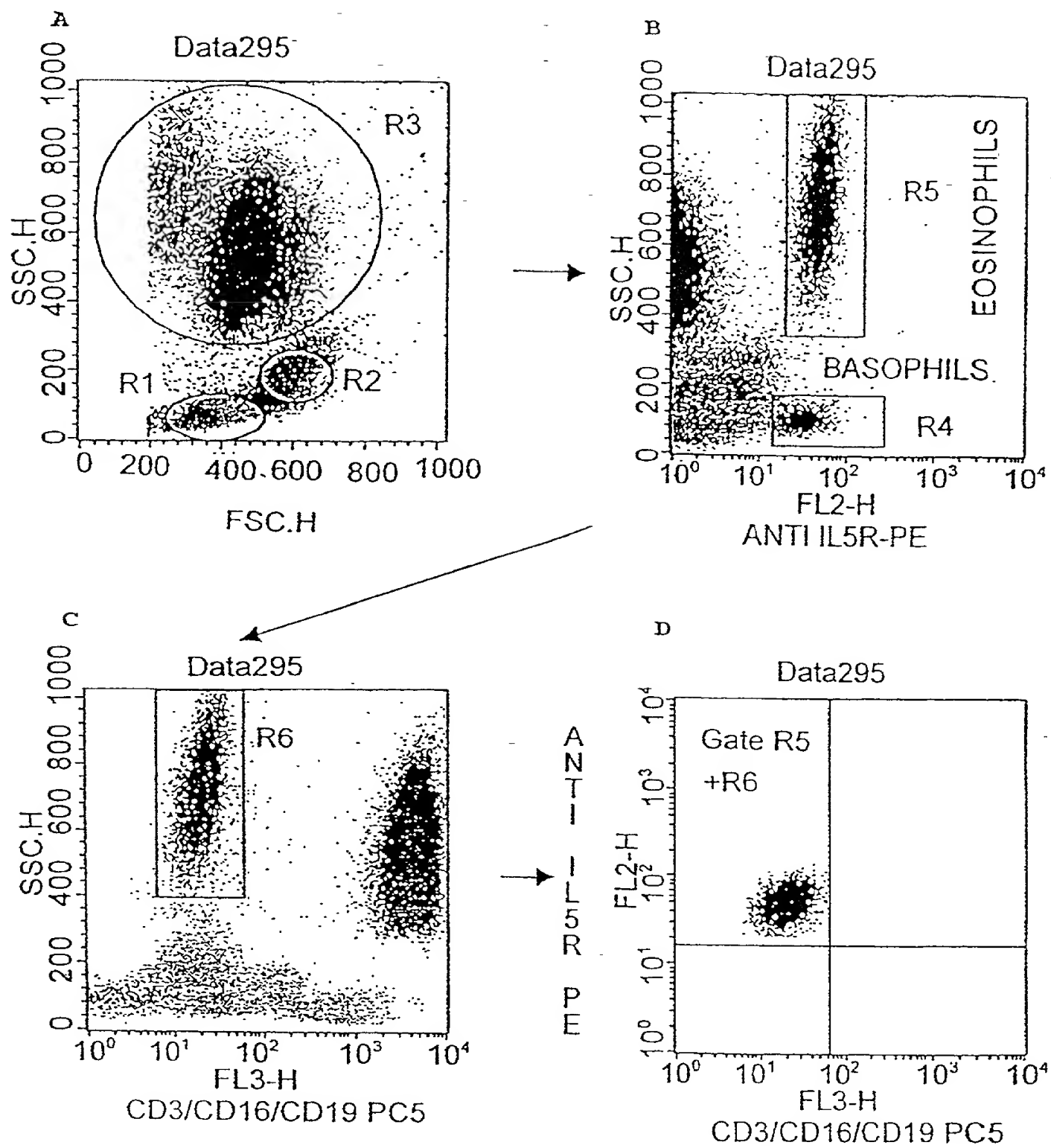


Fig. 3

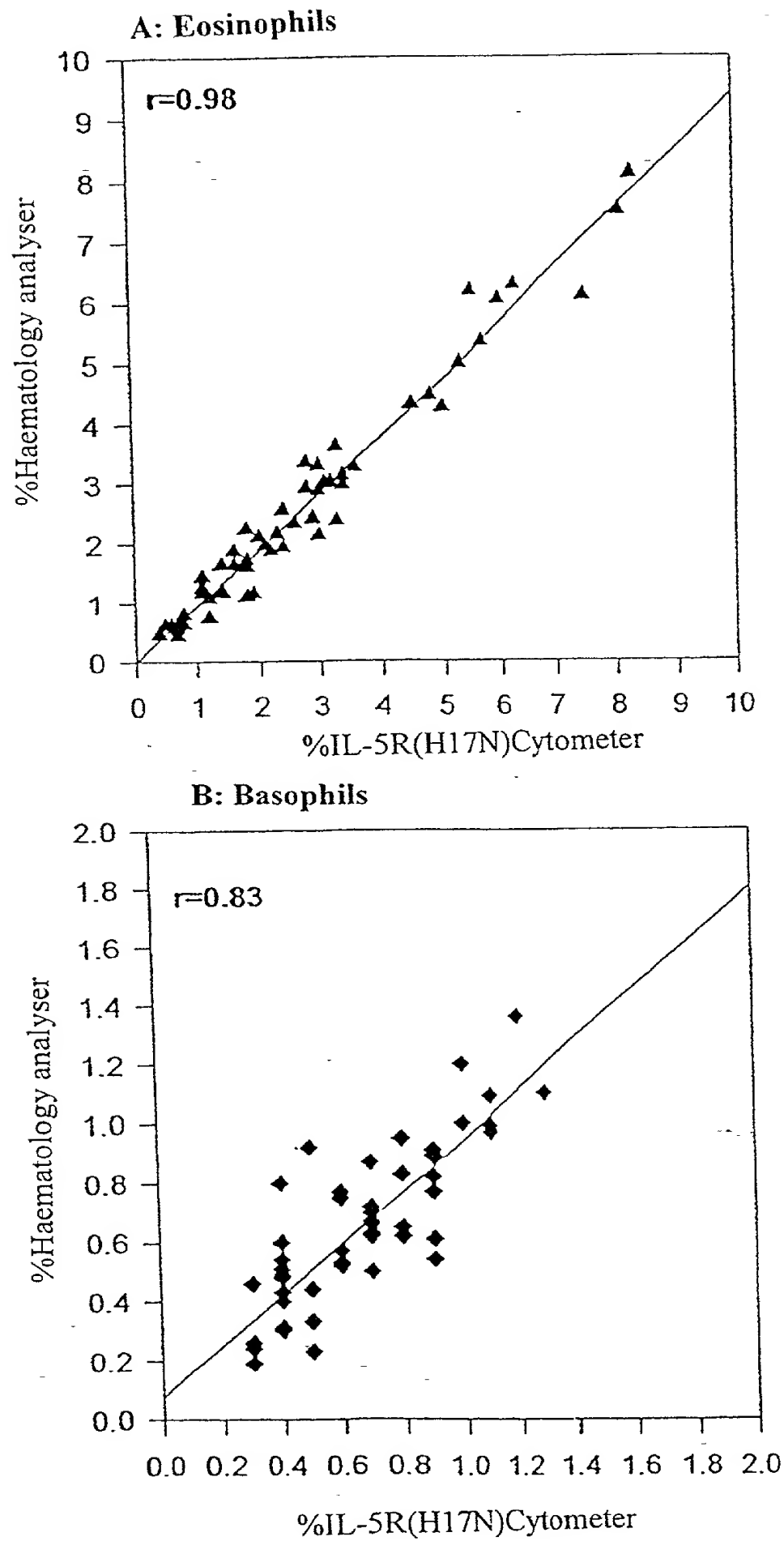


Fig. 4

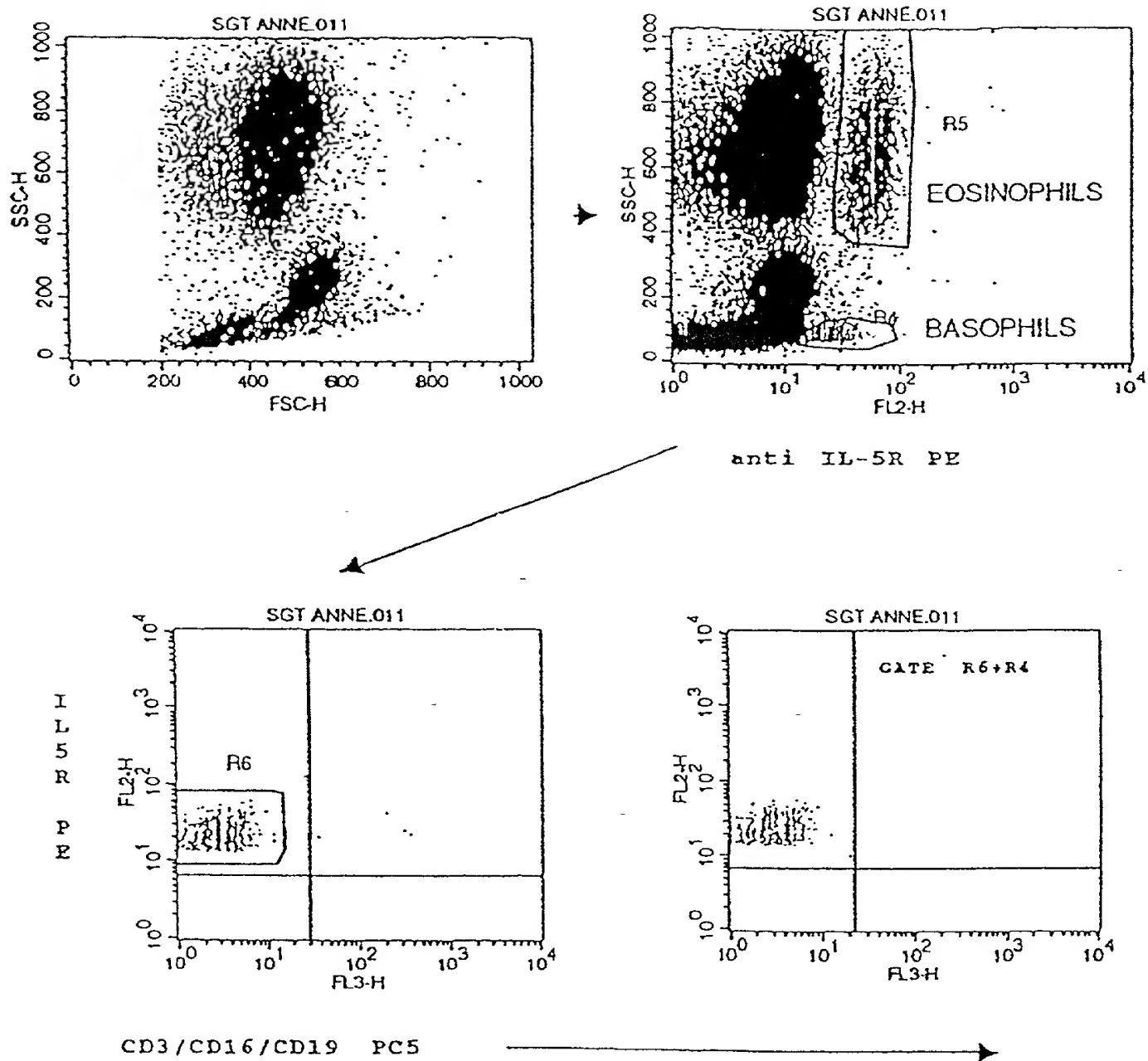


Fig. 5 A

7/9

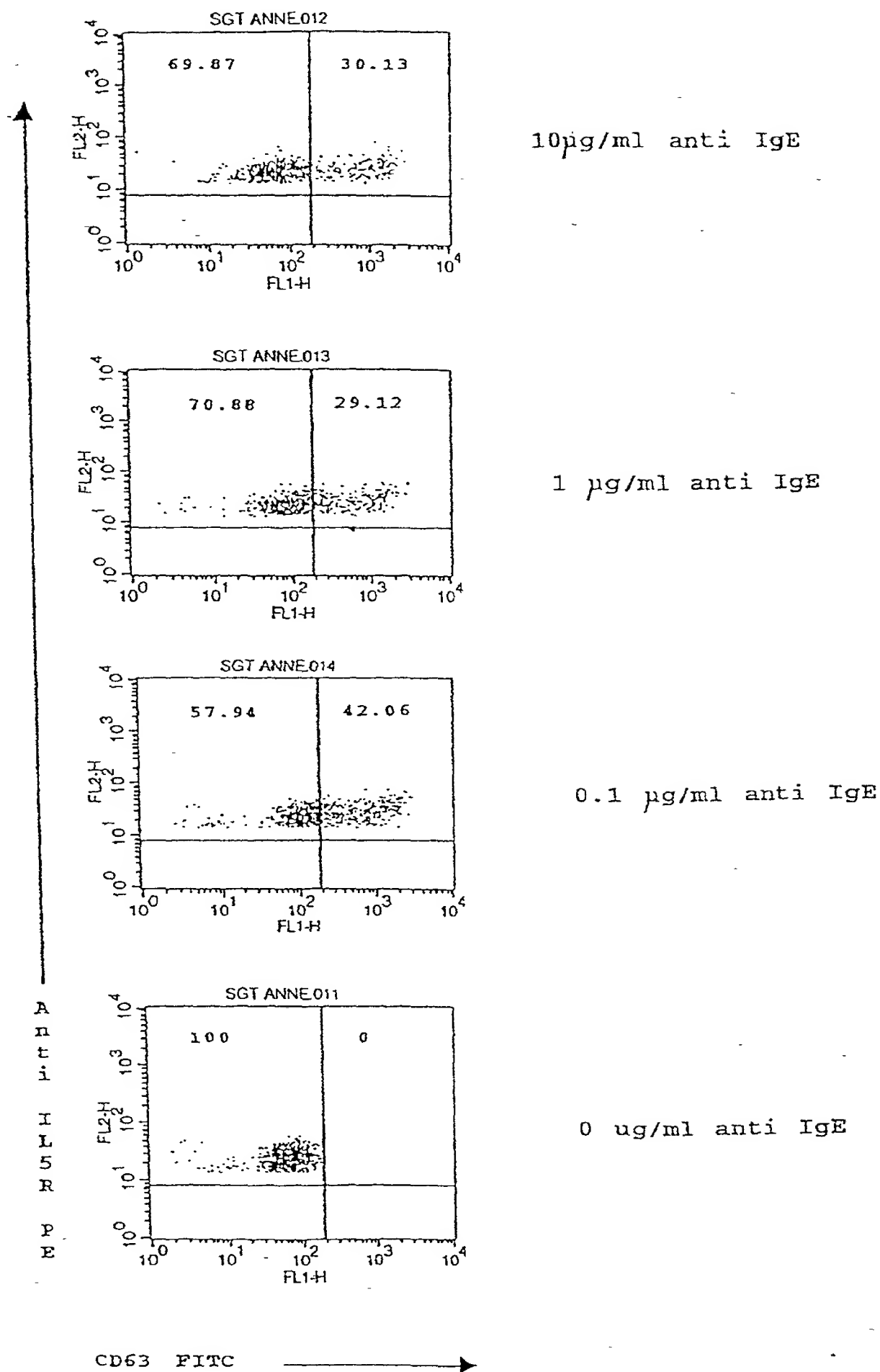


Fig. 5 B

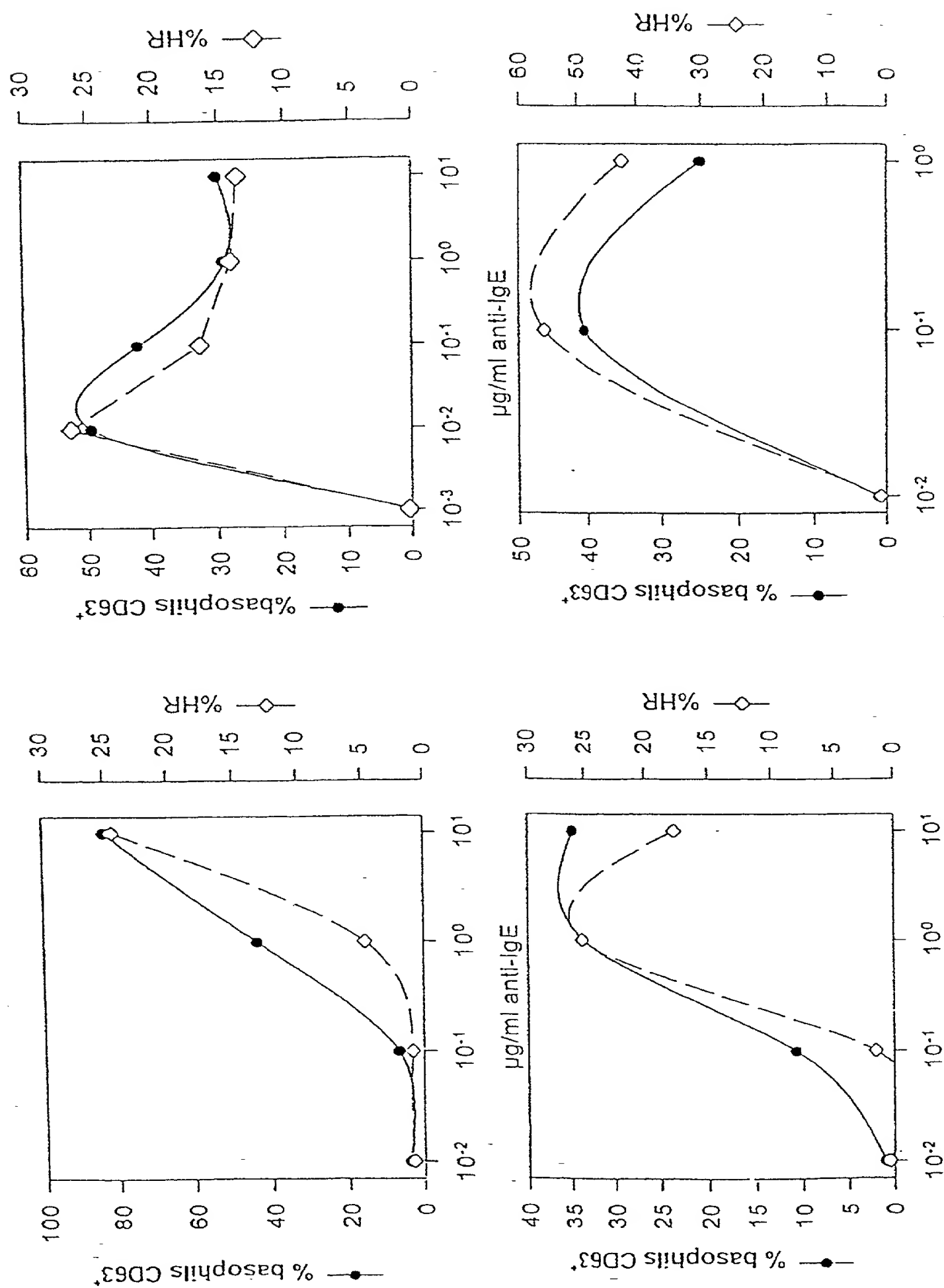


Fig. 6

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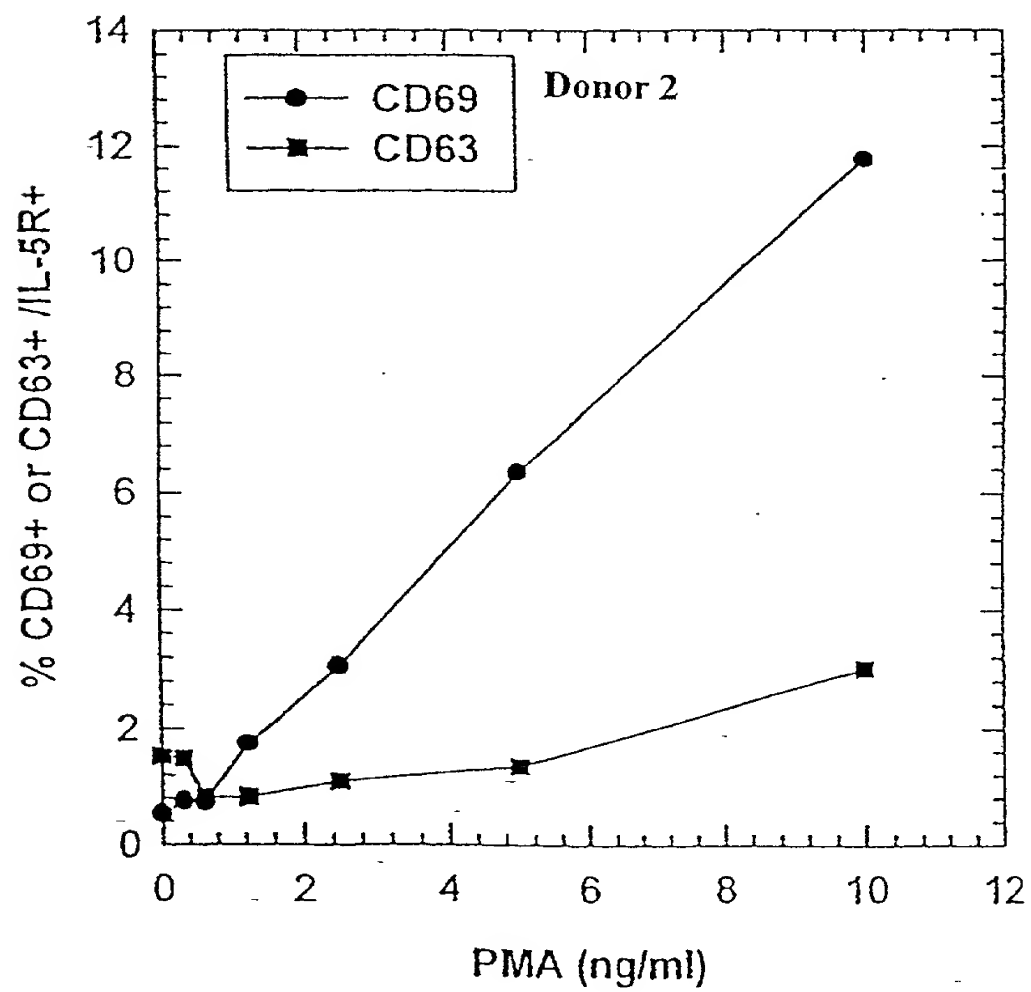
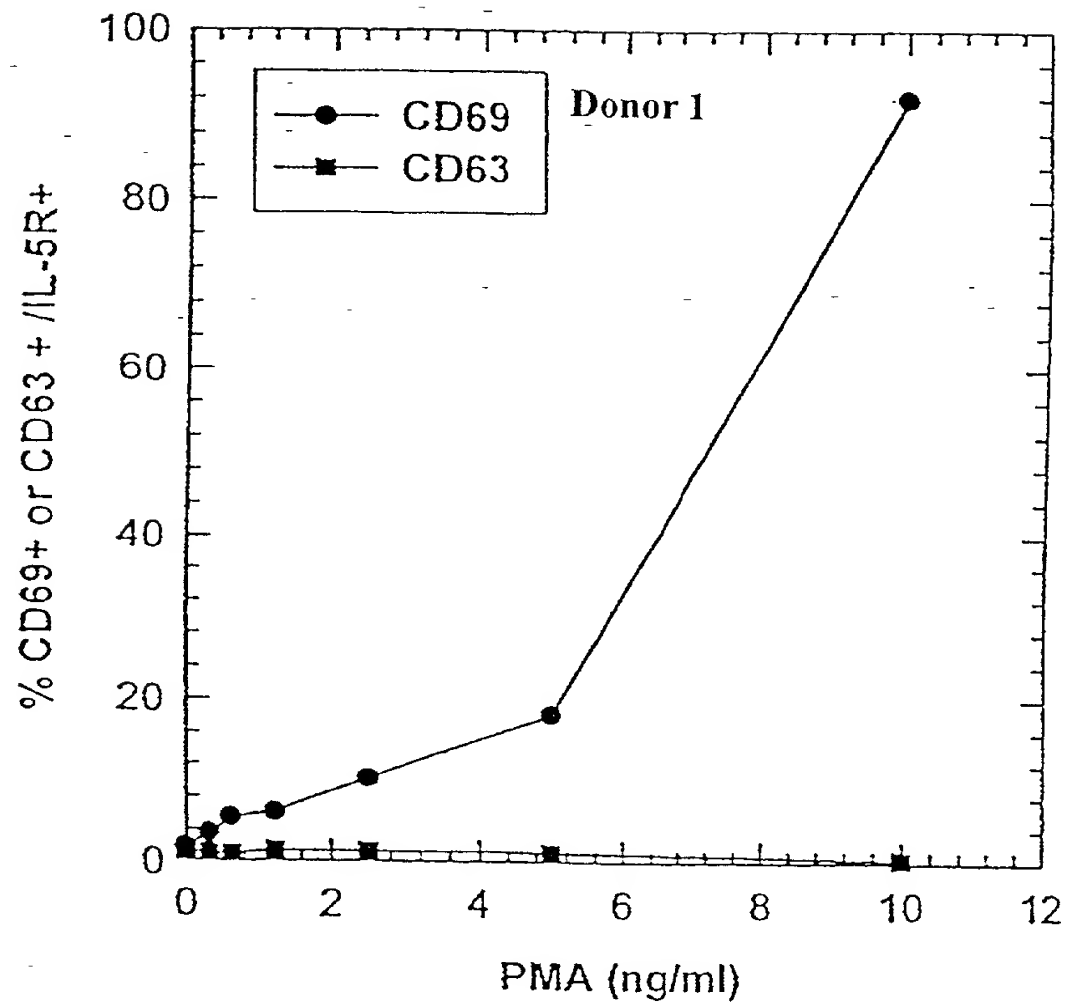


Fig. 7



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Page 1 of 2 Pages

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Atty. Docket: JULIAN1

**Combined Declaration for Patent Application and Power of Attorney**

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and that I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

METHOD FOR DETECTING OR QUANTIFYING BASOPHILS AND EOSINOPHILS

the specification of which (check one)

- ☐ is attached hereto;  
☐ was filed in the United States under 35 U.S.C. §111 on \_\_\_\_\_, as  
 U.S. Appl. No. \_\_\_\_\_\*; or  
☒ was/will be filed in the U.S. under 35 U.S.C. §371 by entry into the U.S. national stage of an international  
 (PCT) application, PCT/FR99/02145; filed September 9, 1999, entry requested on \_\_\_\_\_\*;  
 national stage application received U.S. Appl. No. \_\_\_\_\_\*; §371/§102(e) date  
 \_\_\_\_\_\* (\* if known)

and was amended on \_\_\_\_\_ (if applicable).  
*(include dates of amendments under PCT Art. 19 and 34 if PCT)*

I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above; and I acknowledge the duty to disclose to the Patent and Trademark Office (PTO) all information known by me to be material to patentability as defined in 37 C.F.R. §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §§ 119 (a)-(d) and 365 (b) of any prior foreign application(s) for patent or inventor's certificate, or §365(a) of any prior PCT application(s) designating a country other than the U.S., listed below with the "Yes" box checked, and have also identified below, by checking the "No" box, any foreign application for patent or inventor's certificate or PCT international application having a filing date before that of the application on which priority is claimed:

<u>9811456</u>	<u>France</u>	<u>10 September 1998</u>	<input checked="" type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Day Month Year Filed)	YES	NO
<u>          </u>	<u>          </u>	<u>          </u>	<input type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Day Month Year Filed)	YES	NO

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional applications listed below:

<u>          </u>	<u>          </u>
(Application No.)	(Day Month Year Filed)
<u>          </u>	<u>          </u>
(Application No.)	(Day Month Year Filed)

I hereby claim the benefit under 35 U.S.C. §120 of any prior U.S. non-provisional application(s) or under §365(c) of any prior PCT international application(s) designating the U.S., listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in such U.S. or PCT international application in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose to the PTO all information which is material to patentability as defined in 37 C.F.R. §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

<u>          </u>	<u>          </u>	<u>          </u>
(Application No.)	(Day Month Year Filed)	(Status: patented, pending, abandoned)
<u>          </u>	<u>          </u>	<u>          </u>
(Application No.)	(Day Month Year Filed)	(Status: patented, pending, abandoned)

As a named inventor, I hereby appoint the following registered practitioners to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

**All of the practitioners associated with Customer Number 001444**

Direct all correspondence to the address associated with Customer Number 001444, which is presently:

**BROWDY AND NEIMARK, P.L.L.C.**  
 624 Ninth Street, N.W.  
 Washington, D.C. 20001-5303  
 (202) 628-5197

The undersigned hereby authorizes the U.S. Attorneys or Agents appointed herein to accept and follow instructions from Rinuy, Santarelli as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. Attorneys or Agents and the undersigned. In the event of a change of the persons from whom instructions may be taken, the U.S. Attorneys or Agents appointed herein will be so notified by the undersigned.

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Page 1 of 2 Pages [X] Original [ ] Substitute [ ] Supplemental

Atty. Docket: JULIANI

**Combined Declaration for Patent Application and Power of Attorney**

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and that I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

METHOD FOR DETECTING OR QUANTIFYING BASOPHILS AND EOSINOPHILS

the specification of which (check one)

- ☐ is attached herein;
- ☐ was filed in the United States under 35 U.S.C. §111 on \_\_\_\_\_, as U.S. Appl. No. \_\_\_\_\_; or
- ☒ was/will be filed in the U.S. under 35 U.S.C. §371 by entry into the U.S. national stage of an international (PCT) application, PCT/TR99/02145, filed September 9, 1999, entry requested on \_\_\_\_\_; national stage application received U.S. Appl. No. \_\_\_\_\_; §371/§102(e) date \_\_\_\_\_ ("if known")

and was amended on \_\_\_\_\_ (if applicable).

(include dates of amendments under PCT Art. 19 and 34 (if PCT))

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<u>9811456</u>	<u>France</u>	<u>10 September 1998</u>	<input checked="" type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Day Month Year Filed)	YES	NO
<u>          </u>	<u>          </u>	<u>          </u>	<input type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Day Month Year Filed)	YES	NO

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<u>          </u>	<u>          </u>
(Application No.)	(Day Month Year Filed)
<u>          </u>	<u>          </u>
(Application No.)	(Day Month Year Filed)

I hereby claim the benefit under 35 U.S.C. §120 of any prior U.S. non-provisional application(s) or under §365(c) of any prior PCT international application(s) designating the U.S., listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in such U.S. or PCT international application in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose to the PTO all information which is material to patentability as defined in 37 C.F.R. §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

<u>          </u>	<u>          </u>	<u>          </u>
(Application No.)	(Day Month Year Filed)	(Status: patented, pending, abandoned)
<u>          </u>	<u>          </u>	<u>          </u>
(Application No.)	(Day Month Year Filed)	(Status: patented, pending, abandoned)

As a named inventor, I hereby appoint the following registered practitioners to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

All of the practitioners associated with Customer Number 001444

Direct all correspondence to the address associated with Customer Number 001444, which is presently:

**BROWDY AND NEIMARK, P.L.L.C.**  
 624 Ninth Street, N.W.  
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 (202) 628-5197

The undersigned hereby authorizes the U.S. Attorneys or Agents appointed herein to accept and follow instructions from Rinuy Santarelli as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. Attorneys or Agents and the undersigned. In the event of a change of the persons from whom instructions may be taken, the U.S. Attorneys or Agents appointed herein will be so notified by the undersigned.

Page 2 of 2 Pages

Aity. Docket: JULIANI

Title: METHOD FOR DETECTING OR QUANTIFYING BASOPHILS AND EOSINOPHILS

U.S. Application filed March 12, 2001, Serial No. 09/787,006

PCT Application filed September 9, 1999, Serial No. FR99/02145

I heroby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00	FULL NAME OF FIRST INVENTOR <b>Felix MONTERO JULIAN</b>	INVENTOR'S SIGNATURE: <i>Felix Montero Julian</i>	DATE <b>July 15, 2001</b>
	RESIDENCE <b>Marseille, France</b> <i>JRX</i>	CITIZENSHIP <b>France</b>	
	POST OFFICE ADDRESS <b>6, rue Marie-Louise, Le Marie-Louise, Bât C, 13008 Marseille, France</b>		
2-00	FULL NAME OF SECOND JOINT INVENTOR <b>Anne M. MOREL MONTERO</b>	INVENTOR'S SIGNATURE: <i>Anne MOREL MONTERO</i>	DATE <b>July 15, 2001</b>
	RESIDENCE <b>Marseille, France</b> <i>JRX</i>	CITIZENSHIP <b>France</b>	
	POST OFFICE ADDRESS <b>6, rue Marie Louise, Le Marie-Louise, Bât C, 13008 Marseille, France</b>		
3-00	FULL NAME OF THIRD JOINT INVENTOR <b>Hervé BRAILLY</b>	INVENTOR'S SIGNATURE: <i>Hervé BRAILLY</i>	DATE <b>August 20, 2001</b>
	RESIDENCE <b>Roquevaire, France</b> <i>JRX</i>	CITIZENSHIP <b>France</b>	
	POST OFFICE ADDRESS <b>L'amandière Le Clos, 13360 Roquevaire, France</b>		
	FULL NAME OF FOURTH JOINT INVENTOR <b>Michel DELAGE</b>	INVENTOR'S SIGNATURE	DATE
	RESIDENCE <b>Marseille, France</b>	CITIZENSHIP <b>France</b>	
	POST OFFICE ADDRESS <b>16 rue Adolphe Thiers, 13001 Marseille, France</b>		
	FULL NAME OF FIFTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
	RESIDENCE	CITIZENSHIP	
	POST OFFICE ADDRESS		
	FULL NAME OF SIXTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
	RESIDENCE	CITIZENSHIP	
	POST OFFICE ADDRESS		
	FULL NAME OF SEVENTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
	RESIDENCE	CITIZENSHIP	
	POST OFFICE ADDRESS		

ALL INVENTORS MUST REVIEW APPLICATION AND DECLARATION BEFORE SIGNING. ALL ALTERATIONS MUST BE INITIALED AND DATED BY ALL INVENTORS PRIOR TO SIGNATURE. NO ALTERATIONS CAN BE MADE AFTER THE DECLARATION IS SIGNED. ALL PAGES OF DECLARATION MUST BE SIGNED BY ALL INVENTORS.

Page 1 of 2 Pages

Atty. Docket: JULIANI

Title: METHOD FOR DETECTING OR QUANTIFYING BASOPHILS AND EOSINOPHILSU.S. Application filed March 12, 2001, Serial No. 09/787,006PCT Application filed September 2, 1999, Serial No. FR99/02145

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

FULL NAME OF FIRST INVENTOR <b>Felix MONTERO JULIAN</b>	INVENTOR'S SIGNATURE X	DATE
RESIDENCE <b>Marseille, France</b>	CITIZENSHIP <b>France</b>	
POST OFFICE ADDRESS <b>6, rue Marie-Louise, Le Marie-Louise, Bât C, 13008 Marseille, France</b>		
FULL NAME OF SECOND JOINT INVENTOR <b>ANNE M. MOREL MONTERO</b>	INVENTOR'S SIGNATURE X	DATE
RESIDENCE <b>Marseille, France</b>	CITIZENSHIP <b>France</b>	
POST OFFICE ADDRESS <b>6, rue Marie Louise, Le Marie-Louise, Bât C, 13008 Marseille, France</b>		
FULL NAME OF THIRD JOINT INVENTOR <b>Hervé BRAILLY</b>	INVENTOR'S SIGNATURE X	DATE
RESIDENCE <b>Roquevaire, France</b>	CITIZENSHIP <b>France</b>	
POST OFFICE ADDRESS <b>L'antandière Le Clos, 13360 Roquevaire, France</b>		
FULL NAME OF FOURTH JOINT INVENTOR <b>Michel DELAAGE</b>	INVENTOR'S SIGNATURE X <i>M. Delaage</i>	DATE <b>August 20, 2001</b>
RESIDENCE <b>Marseille, France</b> <i>JRX</i>	CITIZENSHIP <b>France</b>	
POST OFFICE ADDRESS <b>16 rue Adolphe Thiers, 13001 Marseille, France</b>		
FULL NAME OF FIFTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
RESIDENCE	CITIZENSHIP	
POST OFFICE ADDRESS		
FULL NAME OF SIXTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
RESIDENCE	CITIZENSHIP	
POST OFFICE ADDRESS		
FULL NAME OF SEVENTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
RESIDENCE	CITIZENSHIP	
POST OFFICE ADDRESS		

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TOTAL PAGE(S) 02